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Cationic lipids and surfactants as antifungal agents: mode of action

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***Objectives*: To determine the mechanism of antimicrobial action for cationic lipid dioctadecyldimethylammonium bromide (DODAB) and hexadecyltrimethylammonium bromide (CTAB) against *Candida albicans*.**

***Methods*: Determination of DODAB or CTAB adsorption isotherms; cell viability; cell electrophoretic mobility (EM); and leakage of small phosphorylated compounds, proteins or DNA from fungus or haemoglobin from erythrocytes.**

***Results*: High affinity isotherms for CTAB and DODAB adsorption onto fungus cells (10^8 cfu/mL) yield limiting adsorption at 7.8 and 3.7 × 10^9 molecules per cell, respectively. Negatively charged *C. albicans* cells (10^6 cfu/mL) remain viable whereas positively charged ones die. At 0.3 mM CTAB or 0.01 mM DODAB, EM is zero and fungus viability is 50%. Cells start to die at submicellar CTAB concentrations and fungus lysis does not play a significant role in the mechanism of antifungal action. Over 0.1–10 mM CTAB or DODAB, there is no leakage of tested compounds from *C. albicans* cells despite the low cell viability. In contrast to the fungus, under isotonic conditions, cationic amphiphiles induce haemolysis over a range of low DODAB (>0.01 mM) and CTAB (>0.001 mM) concentrations.**

***Conclusions*: The critical phenomenon determining antifungal effect of cationic surfactants and lipids is not cell lysis but rather the change of cell surface charge from negative to positive.**

Keywords: *Candida albicans*, dioctadecyldimethylammonium bromide, hexadecyltrimethylammonium bromide, CTAB, adsorption, cell charge, lysis, antifungal activity

Introduction

Among the classical cationic surfactants, quaternary ammonium compounds (QACs) are the most useful antiseptics and disinfectants.[1,2] QACs are membrane active agents[3–5] and cause lysis of spheroplasts and protoplasts suspended in sucrose.[4,6–9] The cationic agents hypothetically react with phospholipid components in the cytoplasmic membrane, thereby producing membrane distortion and protoplast lysis under osmotic stress.[10,11] On the other hand, the positive charge on microbial cells has often been correlated to the biocidal action.[12–24] The deposition of organic monolayers onto solid surfaces containing quaternary ammonium groups has been shown to prevent deposition and growth of bacterial biofilms.[12] Molecules with a net positive charge are able to kill microorganisms both in solution[12–16] and upon attachment or adsorption to surfaces, particles, liposomes or bilayers.[17–24] Various cationic architectures have been tested such as polyelectrolyte layers[22,25,26] and hyperbranched dendrimers.[27–29]

Over the past decade, our group has been describing the anti-infective properties of cationic bilayers composed of the synthetic lipid dioctadecyldimethyl ammonium bromide (DODAB).[17–21,30–33] Adsorption of DODAB cationic bilayers onto bacterial cells changes the sign of the cell surface potential from negative to positive with a clear relationship between positive charge on bacterial cells and cell death.[30] Regarding the mechanism of DODAB action, neither bacterial cell lysis nor DODAB vesicle disruption takes place.[31] Recently, some polymeric QACs were shown to induce lysis of spheroplasts of *Serratia marcescens*, but not those of *Candida albicans*.[34] In this work, the compared effect of DODAB and hexadecyltrimethylammonium bromide (CTAB) on *C. albicans* cells or erythrocytes is evaluated from adsorption isotherms, cell viability, electrophoretic mobility (EM) and fungus cell lysis as compared with haemolysis. The results clearly show that the critical phenomenon determining antifungal effect of cationic surfactants and lipids is not cell lysis but rather the reversal of cell surface charge from negative to positive.

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Materials and methods

Chemicals and reagents

DODAB was obtained from Sigma Chemical Co. and used as such without further purification. Hexadecyltrimethylammonium bromide or cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) were from Aldrich Chemical Company, Inc. and used as received. Chemical structures and aggregate representations of lipids and surfactants are shown in Figure 1. All other reagents were of analytical grade. Water was Milli-Q quality.

Organism and culture conditions

C. albicans ATCC 90028 (American Type Culture Collection) was plated on 4% dextrose Sabouraud agar. After a period of 24 h growth on agar plates, one or two colonies were transferred to 30 mL of Sabouraud broth, and turbidity (625 nm) was adjusted to 0.5 (McFarland scale). Thereafter, the suspension was incubated in a shaker (30°C/160 rpm/6 h), centrifuged (10000 rpm for 10 min) and the pellet resuspended in Milli-Q water. The last procedure was repeated twice before adjusting turbidity to a final cell concentration of $\sim 2 \times 10^6$ or 2×10^7 cfu/mL. Routinely, cell counting and microscopic visualization of the fungus after these procedures were performed in a Neubauer chamber as a control of fungus yeast morphology. Hyphae morphologies were not observed as expected from fungus growth up to the exponential phase and in a culture medium without serum.

Vesicles preparation

Lipids were dispersed in water or isotonic glucose phosphate buffer (IGP, 1 mM potassium phosphate buffer, pH 7.0, supplemented with 287 mM glucose as an osmoprotectant[35]) using procedures that yielded either large and closed vesicles (LV) or bilayer fragments (BF) depending on the dispersion method. Sonication with a tip was the method employed for producing cationic DODAB BF.[36] This procedure dispersed the amphiphile powder in water using a high-energy input, which not only produced bilayer vesicles but also disrupted these vesicles, thereby generating open BF. DODAB was alternatively dispersed by vortexing at 60°C for obtaining large vesicles (LV). DODAB analytical concentration was determined by halide microtitration.[37]

Determination of adsorption isotherms for amphiphiles onto cells

Interaction between cationic amphiphiles and *C. albicans* cells (1×10^8 cfu/mL) was induced by adding the surfactants to the cells. Mixtures were kept at 25°C for 1 h, unless otherwise stated, and centrifuged at 14000 rpm for 1 h to separate cells from amphiphiles. Supernatant was employed for cationic surfactant or lipid analysis.[38] Adsorption was determined from the difference between total amphiphile added and amphiphile determined in the supernatant and expressed as the number of amphiphile molecules adsorbed per cell.

Determination of fungus electrophoretic mobility in the presence of CTAB, SDS, DODAB BF or DODAB LV

Over a range of amphiphile concentrations, CTAB, SDS, DODAB BF or DODAB LV dispersions and *C. albicans* were mixed at 1×10^6 cfu/mL, allowed to interact for 1 h at room temperature and placed in a cuvette to determine yeasts EM. One should note that the

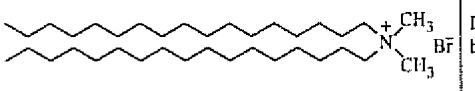
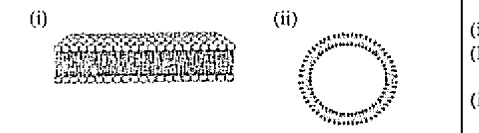
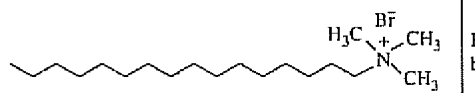
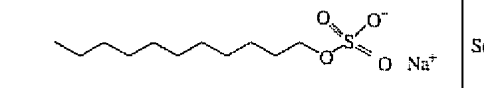
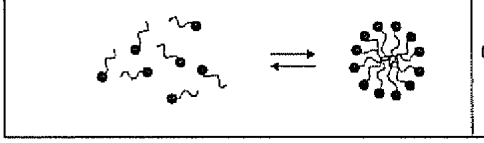
Chemical structures or aggregates	Name
	Dioctadecyldimethylammonium bromide (DODAB)
	(i) Cationic bilayer fragment (DODAB BF) (ii) Large vesicles (DODAB LV)
	Hexadecyltrimethylammonium bromide (CTAB)
	Sodium dodecyl sulphate (SDS)
	CTAB or SDS micelle

Figure 1. Surfactants and lipid chemical structures and aggregates.

measurements were carried out precisely at the same experimental conditions used to obtain the viability curves for *C. albicans* as a function of surfactant concentration. Mobilities were determined using a ZetaPlus Zeta Potential Analyser (Brookhaven Instruments Corporation) and expressed as 10^{-4} cm^2 V^{-1} s^{-1}.

Viability assays in the presence of CTAB, SDS, DODAB BF or DODAB LV

Colony-forming units were counted as a function of CTAB, SDS, DODAB BF or DODAB LV concentration at 1 h of interaction time between *C. albicans* (1×10^6 cfu/mL) and amphiphiles. Plating on agar plates for cfu counts was performed by taking 0.1 mL of a 1000-fold dilution in Milli-Q water of the mixtures. After spreading, plates were incubated for 2 days at 37°C. cfu counts were made using a colony counter.

Determination of leakage of phosphorylated compounds from fungus cells

DODAB-induced leakage of intracellular, low molecular weight, phosphorylated compounds was used as a measurement of cell lysis. SDS and CTAB were also included in the experiment for comparison. Leakage was detected from equilibrium dialysis of the amphiphiles/cell mixture using an equilibrium dialysis chamber with two compartments (*a* and *b*) separated by a cellulose membrane. Six experiments, a, b, c, d, e and f, were performed. In all six experiments, compartment *b* contained 0.9 mL of Milli-Q water. In (a), compartment *a* contained 0.9 mL of NaH_2PO_4 0.15 mM solution. In (b), compartment *a* contained 0.9 mL of *C. albicans* cells (1×10^7 cfu/mL) in Milli-Q water. In other experiments compartment *a* contained 0.9 mL of a mixture of *C. albicans* and CTAB (c), SDS (d), DODAB BF (e) or DODAB LV (f) at different concentrations. Cell lysis was detected and quantified by means of inorganic phosphorus analysis in compartments *a* and *b*.[39]

Release of intracellular material of high molecular weight

Proteins or nucleic acids eventually leaking from cells were determined from light absorption at 260 and 280 nm, respectively, by means of a Hitachi U-2000 spectrophotometer. After incubation with amphiphiles, over a range of experimental conditions, cells (1×10^7 cfu/mL) were pelleted by centrifugation at 5000 rpm for 10 min (Eppendorf Microcentrifuge 5402) and absorbance of the supernatants was determined. As a positive control, cells were disrupted by sonication with a tip (six cycles of 1 min), centrifuged as quoted above and separated from their supernatants by centrifugation. For this positive control, absorbances at 260 and 280 nm were 0.590 and 0.390, respectively.

Determination of amphiphile-induced haemolysis

Human erythrocytes from healthy individuals were collected in vacuum tubes containing heparin (final concentration 20.4 U/mL) as anticoagulant. The erythrocytes were harvested by centrifugation for 2 min at 3000 rpm at 25°C and were washed three times in IGP.[35] A stock 1% cell suspension was prepared from the pellet by dilution in IGP. An aliquot (0.5 mL) of this stock suspension and 0.5 mL of amphiphile dispersions were mixed and incubated at 37°C for 30 min and then centrifuged (2 min/3000 rpm/25°C). The supernatants were used for determining absorbance at 450 nm. Percentage of haemolysis was calculated from [(A_{450} of the amphiphile-treated sample–A_{450} of buffer-treated sample)/(A_{450} of Triton X-100-treated sample)] × 100%. Haemolytic activity of a given agent or HC_{50} is the amphiphile concentration required to induce 50% haemolysis. Determination of HC_{50} was performed from plots for percentage of haemolysis as a function of CTAB, SDS, DODAB BF or DODAB LV concentrations. Total haemolytic activity was achieved with 1% Triton X-100.

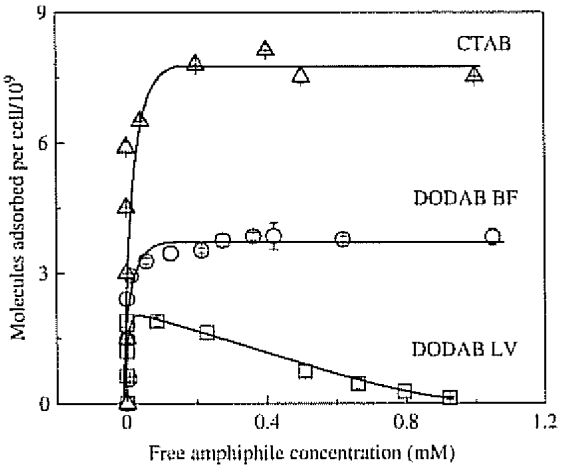


Figure 2. Adsorption isotherms for CTAB, DODAB BF or DODAB LV onto *Candida albicans* (1×10^8 cfu/mL) in Milli-Q water at 25°C. Cells and amphiphile dispersions were left interacting for 1 h before centrifugation and determination of amphiphile concentrations in the supernatant.

Results and discussion

Figure 1 shows amphiphile chemical structure and their assemblies in aqueous solution. Mean diameter and zeta-potentials for DODAB BF are 79 nm and 41 mV whereas for DODAB LV they are ~500–800 nm and 48 mV, respectively.[36] Therefore, cationic aggregates increase in size from the CTAB micelle up to the DODAB LV. As the size of the cationic aggregate increases, adsorption of the cationic amphiphiles decreases (Figure 2). High affinity, langmuirian adsorption isotherms for CTAB and DODAB BF present limiting adsorption of 7.8 and 3.7×10^9 molecules per cell, respectively (Figure 2). Apparently, individual cationic molecules and their small aggregates penetrate more deeply in the dense forest of biomolecules on the cell surface than the large DODAB vesicles. The adsorption isotherm for DODAB LV exhibits a different shape where desorption of LV from the cells takes place as free DODAB concentration increases (Figure 2). For DODAB LV, there is a high affinity adsorption isotherm with a maximum followed by a decrease in adsorption (Figure 2). Possibly, this is due to a significant intervesicle interaction of hydrophobic nature between adsorbed DODAB LV and free DODAB LV so that a competition takes place with cell surface and free vesicles as adsorbents available for LV adsorption. One should recall that DODAB bilayer assemblies are much more stable than micellar CTAB aggregates as depicted from critical micelle concentration (CMC) values for micelle-forming surfactants (10^{-2}–10^{-5} M) and bilayer-forming lipids (10^{-6}–10^{-10} M).[40] The residence time of a monomer in the aggregate is 10^{-1} s in micelles and 10^{+1} s in bilayers, suggesting that exchange rates should fall by eight orders of magnitude from micelles to bilayers.

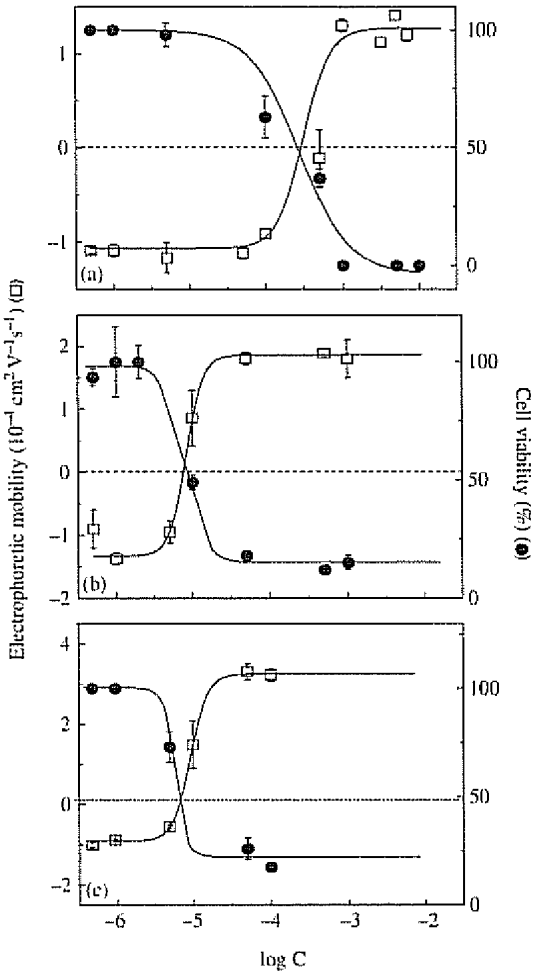


Figure 3. EM and cell viability for *Candida albicans* cells at ~1 × 10^6 cfu/mL as a function of amphiphile molar concentration. Mixtures of cells and CTAB (a), DODAB BF (b) or DODAB LV (c) interacted for 1 h before dilution and plating or EM measurements. Plating on agar (0.1 mL) was performed after a 1:1000 dilution of the mixture.

Figure 3 shows the effect of CTAB (Figure 3a), DODAB BF (Figure 3b) or DODAB LV (Figure 3c) on EM of fungus cells (squares) and on fungus cell viability (filled circles). A correlation between positive EM for the fungus cells and low cell viability is clearly obtained. Over a range of CTAB or DODAB concentrations, the dependence of EM or cell viability on cationic amphiphile concentration is fitted by a sigmoidal function. When EM = 0, there is 50% of cell viability in the presence of 0.3 or 0.01 mM CTAB or DODAB, respectively (Table 1). Thus CTAB is effective regarding killing of fungus cells well below its CMC, which is ~1 mM in 1 mM monovalent salt.[41,42] Usually, the disruptive effect of micelle-forming compounds on cell membranes has been associated to the formation of mixed micelles containing both the surfactant and the cell membrane lipids,[41,42] a phenomenon that occurs at and above the surfactant CMC. Interestingly enough, fungus killing due to CTAB is significant below the CMC suggesting that cell lysis may not be related to fungus killing at such submicellar concentrations. These data agree with those obtained by Ahlström *et al.*,[43] who studied the

Table 1. CTAB, DODAB BF and DODAB LV concentrations at EM = 0 or charge neutralization of *Candida albicans* cell surface charge (cell concentration is 1 × 10^6 cfu/mL)

Amphiphile or lipid dispersion	Concentration of cationic compound (mM)	
	50% viability	EM = 0
CTAB	0.3	0.3
DODAB BF	0.01	0.01
DODAB LV	0.03	0.01

Table 2. Electrophoretic mobility (EM) and *C. albicans* viability evaluated after 1 h of interaction at 25°C with 1 mM CTAB, DODAB BF or DODAB LV before and after washing with Milli-Q water

Sample	EM before	EM after	Viability before (%)	Viability after (%)
Cells	−1.6 ± 0.1	−1.6 ± 0.1	100	100
Cells/CTAB	1.3 ± 0.1	−1.1 ± 0.1	0	0
Cells/DODAB BF	1.8 ± 0.3	1.4 ± 0.1	15 ± 3	9 ± 3
Cells/DODAB LV	3.2 ± 0.1	3.3 ± 0.2	18 ± 1	10 ± 3

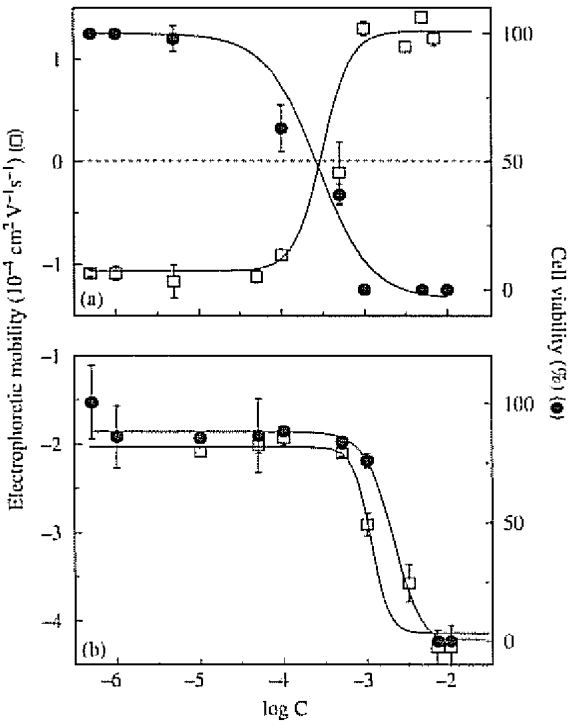


Figure 4. EM and cell viability for *Candida albicans* cells at ~1 × 10^6 cfu/mL as a function of amphiphile molar concentration. Mixtures of cells and CTAB (a) or SDS (b) interacted for 1 h before dilution and plating or EM measurements. Plating on agar (0.1 mL) was performed after a 1:1000 dilution of the mixture.

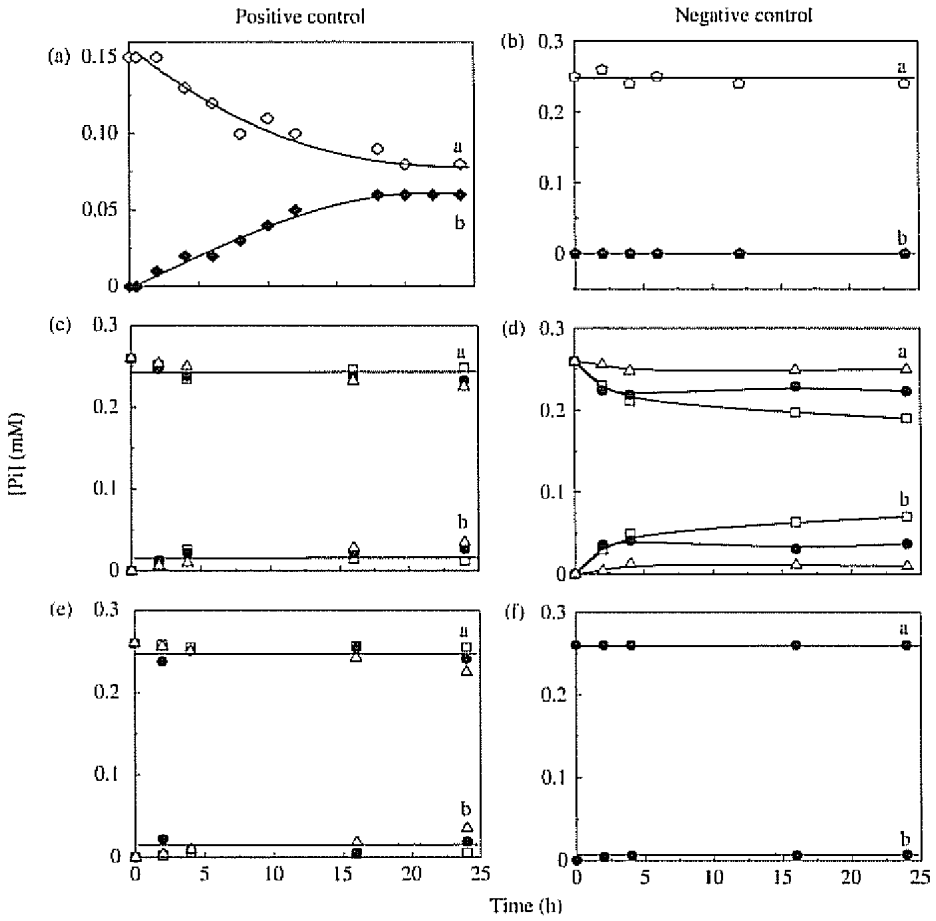


Figure 5. Effect of amphiphile type and concentration on lysis of *Candida albicans* cells. Lysis was evaluated from inorganic phosphorus analysis in hemichambers *a* and *b* of a equilibrium dialysis chamber as a function of time. Compartment *a* contains either NaH_2PO_4 0.15 mM solution (a), *Candida albicans* cells (1×10^7 cfu/mL) (b), or a mixture of cells and CTAB (c), SDS (d), DODAB BF (e) or DODAB LV (f) at 0.1 (open triangles), 1 (filled circles) or 10 mM (open squares) CTAB or DODAB, or 0.7 (open triangles), 7 (filled circles) or 70 mM (open squares) SDS.

killing of *C. albicans* by a series of QACs with different hydrocarbon chain lengths and found that the binding of the compounds to the cells at submicellar concentrations initiated cell killing. Of the compounds tested, hexadecyltrimethylammonium bromide [cetyltrimethylammonium bromide (CTAB)] bound most efficiently due to its higher hydrophobic–hydrophilic balance as compared with the other QACs tested.[43] The present results for DODAB, a molecule which has a larger hydrophobic moiety than CTAB, follow the same trend since the DODAB concentration required to kill 50% of *C. albicans* cells is much smaller than the required CTAB concentration (Figure 3 and Table 1). Apparently the larger hydrophobic moiety of the DODAB molecule causes its efficacy at micromolar concentrations in contrast to the antifungal activity for CTAB observed only over the millimolar range of concentrations (Figure 3 and Table 1).

In contrast to CTAB (Figure 3a), cell viability in the presence of DODAB never reached 0%, attaining at most 10%. As previously suggested for bacteria[17] and fungi,[44,45] cell aggregation increases as a function of cell concentration and might be considerable at the cell concentration employed for testing cell viability in this work (1×10^6 cfu/mL). As previously observed for bacteria,[17] the number of large DODAB vesicles adsorbed per cell decreased as a function of cell number density (n), possibly owing to increased cell aggregation and reduction of the total cell area available for adsorption with n.[17] Possibly, the cells which remain viable are those inside cell aggregates that cannot be reached by DODAB BF or LV. One should note, however, that aggregated cells are apparently defenceless in front of CTAB individual molecules, which apparently penetrate more easily and deeply inside *C. albicans* aggregates determining 0% cell viability at and above 1 mM CTAB (Figure 3a).

The value given for CMC of CTAB as well as the results of 50% killing are shown at ~10 times higher concentration in this paper compared with those given in reference 43. A possible explanation for the difference in CMC values can be found from the different ionic strengths. The present work was performed in water whereas Ahlström and coworkers used 10 mM phosphate buffer. Micelle formation is strongly dependent on intermolecular forces acting between CTAB monomers. Upon increasing ionic strength, electrostatic repulsion between monomers decreases causing a decrease in CMC. In addition, CMC of ionic surfactants was often observed to decrease as temperature increases.[42]

This leads to the possibility that the differences in CMC values for CTAB in water at 25°C (this work) and in 10 mM phosphate buffer at 37°C[42] could eventually be due, in part, to the different temperatures. In summary, both differences in temperature and ionic strength could explain the different results obtained by us and Ahlström *et al.*[43] regarding cell killing over a range of CTAB concentrations.

Reversibility of effects of cationic compounds on cell surface charge and viability was also evaluated. Cells (10^6 cells/mL) and cationic compound (1 mM CTAB or DODAB BF or DODAB LV) interacted for 1 h at 25°C, before centrifuging, removing supernatant, resuspending the pelleted cells in pure water and determining mean EM and viability for yeast cells (Table 2). CTAB could be completely removed from cells by one washing step as depicted from the large difference in EMs before and after washing. However, this was not possible for DODAB BF or LV, which remained adsorbed as depicted from the very similar mean EM values obtained after the washing step (Table 2). Cell viability was not altered by washing cells with water despite reversal of cells surface charge after removal of CTAB (Table 2). In the case of cationic DODAB bilayers in the form of BF or LV, DODAB desorption was practically absent upon washing suggesting an intimate and strong interaction between important biomolecules of the cell and the cationic bilayers.

Figure 4 shows the compared effect of CTAB (Figure 4a) and a common anionic surfactant such as SDS (Figure 4b) on fungus cell viability and EM. Whereas the anionic surfactant induced significant leakage of low molecular weight phosphorylated compounds at and above its CMC (Figure 5d) as compared with positive (Figure 5a) or negative controls (Figure 5b), CTAB (Figure 5c), DODAB BF (Figure 5e) and DODAB LV (Figure 5f) did not induce any leakage. Therefore, there seems to be a difference in mechanism of antifungal action for the two classes of amphiphiles. However, one should notice that equilibrium dialysis will be influenced by the CTAB-treated cells, which are now like positively charged particles, and may serve the function of ionic exchange beads. The inorganic phosphorus might have been retained as counter ions and not diffused through the cellulose membrane. This would also be a possible explanation for the absence of phosphorylated compounds in hemichamber *b* of the dialysis chamber.

In order to confirm SDS-induced cell lysis for the fungus and CTAB or DODAB action in absence of lysis, protein and DNA leakage from the cell interior were recorded as a function of cells interaction time with amphiphiles at concentrations 10 times above their CMC. At 70 mM SDS, lysis with appearance of protein and DNA outside the cells is shown, whereas 10 mM CTAB or DODAB BF did not cause any leakage (Figure 6a and b). The same experiment shown in Figure 6, which was performed in pure water, was repeated in 10 or 150 mM of ionic strength in the presence of phosphate buffer at pH 7.0 and similar results were obtained (not shown). Also similar experiments were performed at 0.1 and 1.0 mM CTAB, DODAB BF or DODAB LV yielding absence of UV-absorbing compounds in the supernatants (not shown). Nucleic acids and proteins might be aggregated/precipitated by the cationic compounds and not recovered in the supernatants, especially at the highest concentrations of cationic compounds. Ahlström *et al.*[43] have previously observed a decrease of released UV-absorbing substances already at a concentration of 1 mM CTAB. However, as we have not detected UV-absorbing species in the supernatant at 0.1 mM of

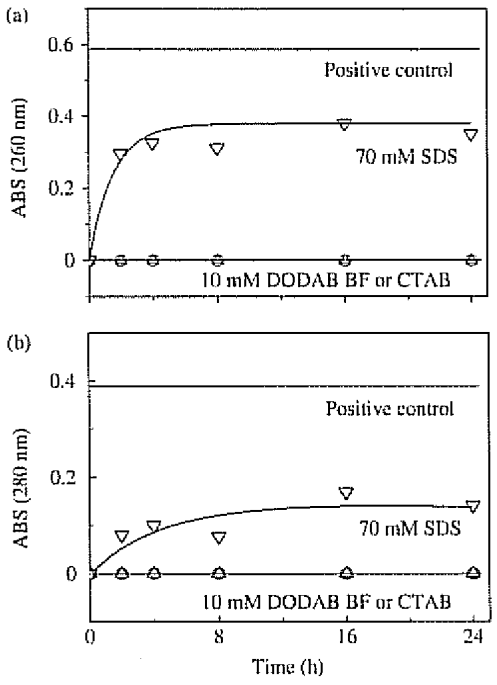


Figure 6. Effect of SDS (open upside-down triangles), CTAB (open triangles) or DODAB BF (open circles) on the release of intracellular material from *Candida albicans* cells at 1×10^7 cfu/mL. Supernatant absorbance at 260 (a) or 280 nm (b) for the detection of DNA or protein, respectively, was determined as a function of incubation time after centrifugation of the mixtures.

Table 3. CTAB or DODAB concentration required for 50% haemolysis of human red blood cells (HC_{50}) at 0.5%

Amphiphile or lipid dispersions	HC_{50} (μM)
CTAB	0.7
DODAB BF	1.4
DODAB LV	4.0

cationic compounds, the hypothesis of absence of cell lysis in the presence of the cationic compounds is reinforced.

In order to evaluate the amphiphiles effect on a different cell, haemolysis of human erythrocytes was determined and quantified from plots of percentage haemolysis as a function of amphiphile concentration. HC_{50} concentrations (concentrations required to cause 50% haemolysis) are shown in Table 3. CTAB or DODAB concentrations required for 50% haemolysis belonged to the micromolar range, in clear contrast to absence of *C. albicans* lysis over a range of tenths of millimolar for the same amphiphiles. This is understandable from the robust character of the *C. albicans* cell wall in comparison with the more fragile cellular surface of erythrocytes and indicates that cationic surfactants and lipids cannot possibly reach the protected cell membrane from the fungus. Otherwise they might hamper the function of essential proteins that act as transporters of nutrients or metabolites to be excreted by the fungus cell.

Conclusions

Over a broad range of concentrations, the mechanism of antifungal action of micelle-forming cationic detergent CTAB or the bilayer-forming cationic synthetic lipid DODAB does not involve fungus cell lysis but rather the change of cell surface charge from negative to positive. Mammalian cells, on the other hand, are much more fragile regarding CTAB or DODAB-induced cell lysis, being disrupted by cationic amphiphiles over a range of micromolar concentrations.

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Transparency declarations

None to declare.

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